Protective effect of 17β-estradiol on ischemic acute renal failure through the PI3K/Akt/eNOS pathway

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Estrogens attenuate renal injury induced by ischemia/reperfusion (I/R), an effect that is related to nitric oxide production in the post-ischemic kidney. The compound 17β-estradiol (E_2-β) acting via estrogen receptors (ERs) is known to activate endothelial nitric oxide synthase (eNOS) through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway. We determined if this pathway contributes to the renoprotective effect of E_2-β in the uninephrectomized ischemia reperfusion rat model of acute renal injury. Treatment with E_2-β suppressed the I/R-induced increases in blood urea nitrogen, plasma creatinine, urine flow, and fractional excretion of sodium while augmenting creatinine clearance, renal blood flow, and urine osmolality, indicating attenuation of renal injury. Phosphorylation of Akt and eNOS protein was significantly increased 30–60 min after reperfusion in estradiol-treated compared to vehicle-treated rats. The protective effects of E_2-β and protein phosphorylation were reversed by the PI3K inhibitor wortmannin or the ER antagonist tamoxifen. Furthermore, the E_2-β-induced renoprotective effects were not seen in eNOS knockout mice with renal injury. We conclude that the E_2-β-induced renoprotective effect is due to activation of the PI3K/Akt pathway followed by increased eNOS phosphorylation in the post-ischemic kidney.

Kidney International (2008) **73,** 308–317; doi:10.1038/sj.ki.5002690; published online 14 November 2007

KEYWORDS: acute renal failure; 17β-estradiol; nitric oxide; phosphatidylinositol-3 kinase; Akt

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Received 5 December 2006; revised 23 August 2007; accepted 28 August 2007; published online 14 November 2007

It is well known that the incidence of cardiovascular diseases is lower in women prior to menopause compared with men and postmenopausal women.[1] Estrogen replacement therapy attenuates the incidence of cardiovascular events in postmenopausal women. Recently, it is becoming increasingly appreciated that gender differences exist in susceptibility to and/or mortality from ischemia/reperfusion (I/R)-induced injuries in heart,[2,3] liver,[4] and kidney.[5,6] There are consistent findings that females are less susceptible to these I/R-induced injuries compared with males. Indeed, Harada *et al.*[4] showed that the protective effect observed in female mice against reduced-size liver I/R injury was attenuated by ovariectomy or administration of an estrogen antagonist. Park *et al.*[5] demonstrated that orchiectomy decreased the kidney susceptibility to I/R-induced acute renal failure (ARF) in male mice. Thus, sex hormones may be the important determinant in these gender differences of various diseases.

The molecular mechanisms underlying the I/R-induced ARF are not fully understood, but several casual factors are contributive to the pathogenesis of this renal lesion.[7] In the post-ischemic kidney, there is increased reactivity to vasoconstrictive agents and decreased vasodilatory responses in arterioles compared with normal kidney.[8] Endothelial dysfunction is an important component of initiating and continuing renal tubular epithelial injury and contributes to the pathogenesis of I/R-induced renal injury.[9] In addition, endothelial injury aggravates the inflammatory response through loss of normal nitric oxide (NO) production due to inhibition of endothelial NO synthase (eNOS).

NO works as an important endogenous modulator of I/R-induced renal injury.[10] NO precursor L-arginine has been reported to ameliorate the post-ischemic ARF.[11] Furthermore, inhibition of NOS was seen to aggravate the renal I/R injury.[12] We have demonstrated that pre-ischemic treatment with FK409, a spontaneous NO releaser, exerted a remarkable protective effect against the post-ischemic ARF.[13] Taken together, NO seems to function as a renoprotective factor against the I/R-induced renal injury.

Recently, we have reported that 17β-estradiol (E_2-β) protects the kidney against the post-ischemic injury in male rats and that the protective effects of E_2-β were markedly attenuated by the pretreatment with N^G-nitro-L-arginine

methyl ester (L-NAME), a non-selective NOS inhibitor.[14] Furthermore, we noted that the renal NO_2^-/NO_3^- (NO_x) level was markedly reduced during ischemia and after reperfusion and that the reduced level recovered rapidly to the basal level in the E_2-β-treated ARF rats compared with the vehicle-treated ARF rats. These findings suggest that E_2-β has a protective effect against the post-ischemic ARF by increasing the production of NO. However, mechanisms underlying E_2-β-induced NO production in the post-ischemic kidney are not elucidated.

E_2-β is known to increase the endothelial NO production, which is contributive to its vasoprotective action. This can be explained at least in part by increases in the eNOS mRNA expression and its protein level. These effects are consistent with the classic genomic mechanism of estrogen receptors (ERs) action that involves transcription of target genes in the nucleus. On the other hand, there is growing evidence that ERs also mediate non-genomic effects by activating cell signaling pathways outside the nucleus. A number of cell culture studies have suggested that E_2-β rapidly increases NO release by stimulating the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, depending on ERs.[15–18]

Thus, the purpose of the present study is to determine whether E_2-β prevents I/R-induced renal injury by stimulating the PI3K/Akt pathway, resulting in the increased production of NO in the post-ischemic kidney.

RESULTS

Effects of E_2-β and wortmannin on renal function after the I/R

As shown in Figure 1, the renal function of rats subjected to 45-min ischemia and reperfusion showed marked deterioration when measured 1 day after reperfusion. As compared with sham-operated rats, vehicle-treated ARF rats showed significant increases in blood urea nitrogen (BUN), creatinine levels in plasma (Pcr), urine flow, and fractional excretion of sodium (FE_{Na}), and significant decreases in creatinine clearance (Ccr) and urinary osmolality (Uosm). Administration of E_2-β to ARF rats 15 min before reperfusion markedly attenuated the I/R-induced renal dysfunction. Pre-ischemic treatment with wortmannin (10 min before ischemia), a PI3K inhibitor, markedly attenuated the protective effects of E_2-β. Wortmannin alone did not affect the renal dysfunction induced by I/R.

Effects of E_2-β and wortmannin on histological renal damage after the I/R

Histological examinations revealed severe lesions in the kidney of vehicle-treated ARF rats (1 day after the 45-min ischemia and reperfusion). These changes were characterized by proteinaceous casts in tubuli in the inner zone of medulla (scores 3.67 ± 0.33), medullary congestion, and hemorrhage in the outer zone inner stripe of medulla (scores 3.33 ± 0.33), and tubular necrosis in the outer zone outer stripe of medulla (scores 3.67 ± 0.33). Treatment with E_2-β of ARF rats attenuated the development of all these lesions, but the combination with wortmannin reversed the protective effect of E_2-β (Figure 2 and Table 1).

Effects of E_2-β and tamoxifen on renal function after the I/R

As shown in Figure 3, the pre-ischemic treatment with tamoxifen (60 min before ischemia), an ERs antagonist, abolished the E_2-β-induced renoprotective effects. In contrast, tamoxifen alone did not affect the renal dysfunction induced by I/R (data not shown).

Effects of E_2-β and tamoxifen on histological renal damage after the I/R

The pre-ischemic treatment with tamoxifen also suppressed the protective effect of E_2-β on histological renal damage after I/R (Table 2).

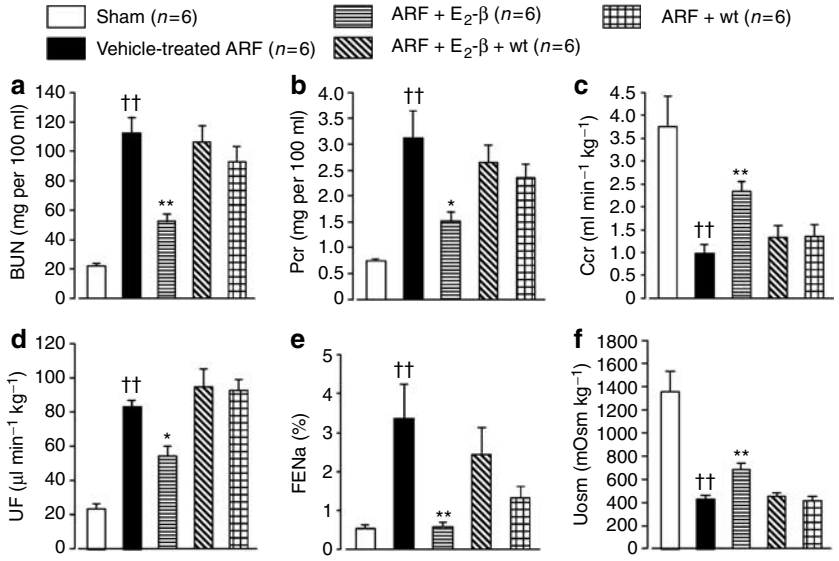

Figure 1 | Effects of E_2-β and wortmannin (wt) on (a) BUN, (b) Pcr, (c) Ccr, (d) urine flow (UF), (e) FE_{Na}, and (f) Uosm 24 h after I/R. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and wortmannin (10 μg kg^{-1}, i.v.) was given 10 min before ischemia. Each column and bar represents the mean ± s.e.m. $^{\dagger\dagger}P<0.01$, compared with sham rats. $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF rats.

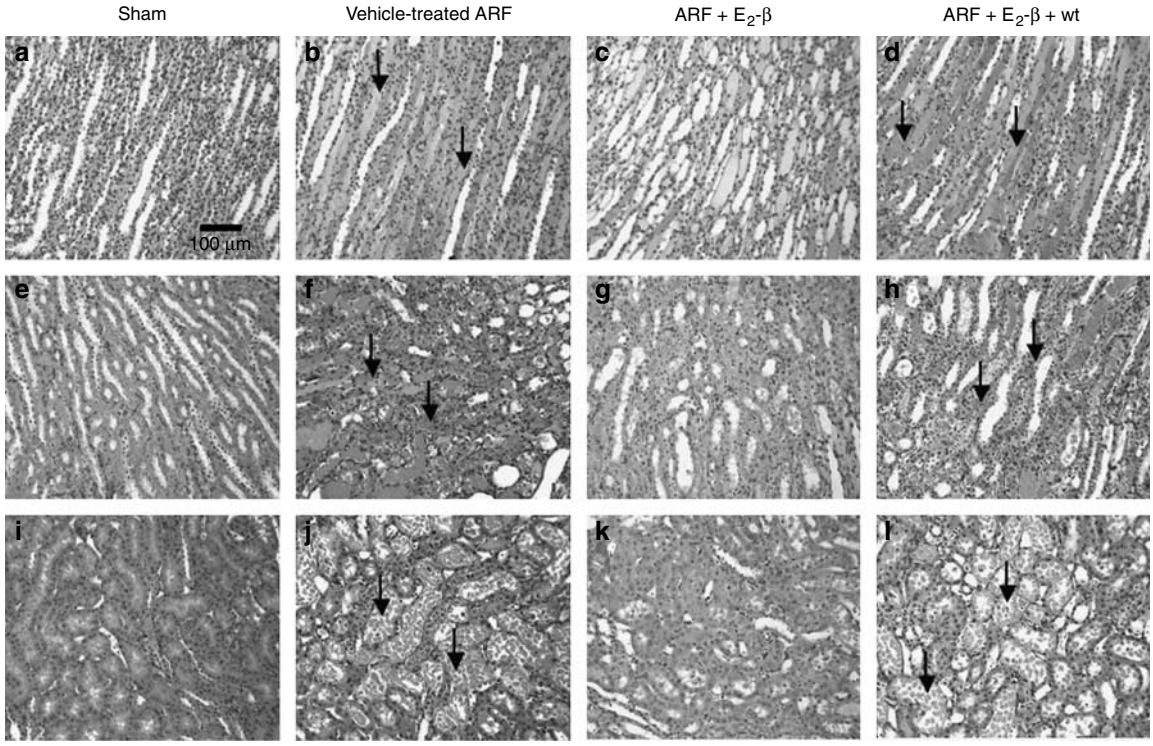

Figure 2 | Light microscopy of (a–d) the inner zone of medulla, (e–h) the outer zone inner stripe, and (i–l) the outer zone outer stripe of kidneys of ARF rats treated with (b, f, and j) vehicle, (c, g, and k) E_2-β or (d, h, and l) E_2-β + wortmannin (wt), and (a, e, and i) sham-operated rats. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and wortmannin (10 μg kg^{-1}, i.v.) was given 10 min before ischemia. Arrows indicate (**b** and **d**) severe proteinaceous casts in tubuli, (**f** and **h**) congestion and hemorrhage, and (**j** and **l**) tubular necrosis (hematoxylin–eosin staining, original magnification × 200).

Table 1 | Effects of E_2-β and wt on histopathological changes in kidneys of ARF rats

	Vehicle-treated ARF (*n*=6)	ARF+E_2-β (*n*=5)	ARF+E_2-β+wt (*n*=6)
Proteinaceous casts in tubuli	3.67 ± 0.33	2.00 ± 0.32*	2.67 ± 0.42
Medullary congestion	3.33 ± 0.33	2.00 ± 0.45*	3.17 ± 0.40
Tubular necrosis	3.67 ± 0.33	1.80 ± 0.20**	3.17 ± 0.31

ARF, acute renal failure; E_2-β, 17β-estradiol; wt, wortmannin.
Each value represents the mean ± s.e.m. of histopathological score. Grades of score: no change (0), mild (1), moderate (2), severe (3), very severe (4). *$P<0.05$, **$P<0.01$, compared with vehicle-treated ARF rats.

Time course of phosphorylated-Akt (Ser^{473}) and phosphorylated-eNOS (Ser^{1177} and Thr^{495}) protein expression in the post-ischemic kidneys with or without E_2-β treatment

Expression of renal phosphorylated-Akt (Ser^{473}) protein tended to increase transiently in the post-ischemic kidney compared with that of sham rat, but thereafter the increased level declined gradually to sham level. In E_2-β-treated ARF rats, there were significant high levels of phosphorylated-Akt protein expression at 10, 30, and 60 min after reperfusion compared with vehicle-treated ARF rats (Figure 4). Furthermore, phosphorylated-eNOS (Ser^{1177}) protein expression was also increased significantly in E_2-β-treated ARF rats in the same manner as described above, compared with that of vehicle-treated ARF rats, in which this protein expression slightly and gradually increased after reperfusion (Figure 5). The phosphorylated-eNOS (Thr^{495}, a negative regulatory site) expression was also increased transiently and slightly in the post-ischemic kidney, but there is no difference with or without E_2-β treatment (data not shown). Neither I/R itself nor E_2-β treatment changed total Akt and eNOS protein levels (Figures 4 and 5).

Time course of constitutive NOS activity in the post-ischemic kidneys with or without E_2-β treatment

Changes of NOS activity were evaluated in kidneys exposed to I/R with or without E_2-β treatment. As shown in Table 3, the constitutive NOS (cNOS) activities in vehicle-treated ARF rats were slightly increased at 60 min after reperfusion compared with sham-operated rats. In contrast, the cNOS activities in E_2-β-treated ARF rats were increased gradually and significant changes were observed at 60 min after reperfusion. Inducible NOS activity was not detected in kidneys of sham-operated rats and ARF rats with or without E_2-β treatment (data not shown).

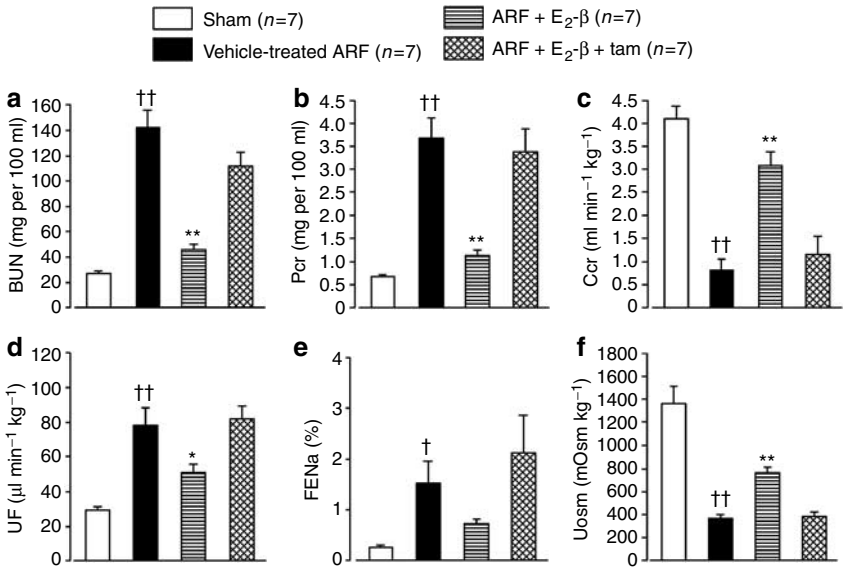

Figure 3 | Effects of E_2-β and tamoxifen (tam) on (a) BUN, (b) Pcr, (c) Ccr, (d) urine flow (UF), (e) FE_{Na}, and (f) Uosm 24 h after I/R. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and tamoxifen (5 mg kg^{-1}, i.v.) was given 60 min before ischemia. Each column and bar represents the mean ± s.e.m. $^{\dagger}P<0.01$, $^{\dagger\dagger}P<0.01$, compared with sham rats. $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF rats.

Table 2 | Effects of E_2-β and tam on histopathological changes in kidneys of ARF rats

	Vehicle-treated ARF (*n*=7)	ARF+E_2-β (*n*=7)	ARF+E_2-β+tam (*n*=4)
Proteinaceous casts in tubuli	3.71 ± 0.29	1.86 ± 0.34**	3.50 ± 0.29
Medullary congestion	3.71 ± 0.29	2.00 ± 0.38**	3.50 ± 0.29
Tubular necrosis	3.86 ± 0.14	2.00 ± 0.22**	3.25 ± 0.25

ARF, acute renal failure; E_2-β, 17β-estradiol; tam, tamoxifen.
Each value represents the mean ± s.e.m. of histopathological score. Grades of score: no change (0), mild (1), moderate (2), severe (3), very severe (4). $^{**}P<0.01$, compared with vehicle-treated ARF rats.

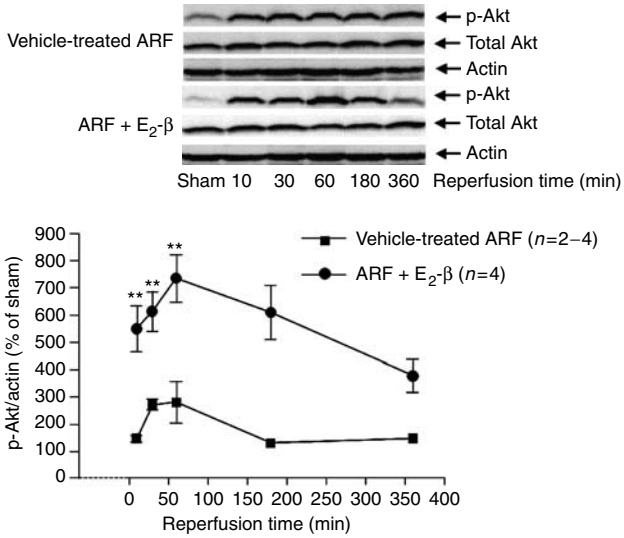

Figure 4 | Time course of total Akt and phosphorylated-Akt (p-Akt, Ser^{473}) expression in the kidney of vehicle-treated or E_2-β-treated ARF rats. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion. Mean density values are presented as fold difference compared with sham-operated rats. Each point and bar represents the mean ± s.e.m. $^{**}P<0.01$, compared with vehicle-treated ARF rats.

Effects of E_2-β, wortmannin, and tamoxifen on expression of phosphorylated-Akt (Ser^{473}) and phosphorylated-eNOS (Ser^{1177}) protein

As shown in Figures 6 and 7, the increases in both phosphorylated-Akt (Ser^{473}) and phosphorylated-eNOS (Ser^{1177}) proteins at 30 and 60 min after reperfusion in E_2-β-treated ARF rats were abolished by pre-ischemic treatment with wortmannin. Similarly, the pre-ischemic treatment with tamoxifen given to ARF rats completely suppressed the E_2-β-induced increments of both proteins at 30 and 60 min after reperfusion (Figures 8 and 9).

Time course of renal blood flow in the post-ischemic kidneys with or without E_2-β treatment

Changes of renal blood flow were evaluated in anesthetized (sodium thiobutabarbital: inactin, 100 mg kg^{-1}, intraperitoneal (i.p.)) rats exposed to I/R with or without E_2-β treatment, using an electromagnetic flow probe (1.0 mm in diameter, Nihon Kohden, Tokyo, Japan) connected to a square-wave flowmeter (MFV-2100; Nihon Kohden). As shown in Figure 10, renal blood flow in the post-ischemic kidneys gradually restored and reached a plateau at 1 h after the start of reperfusion, but E_2-β treatment significantly produced an early recovery at 10–30 min after reperfusion.

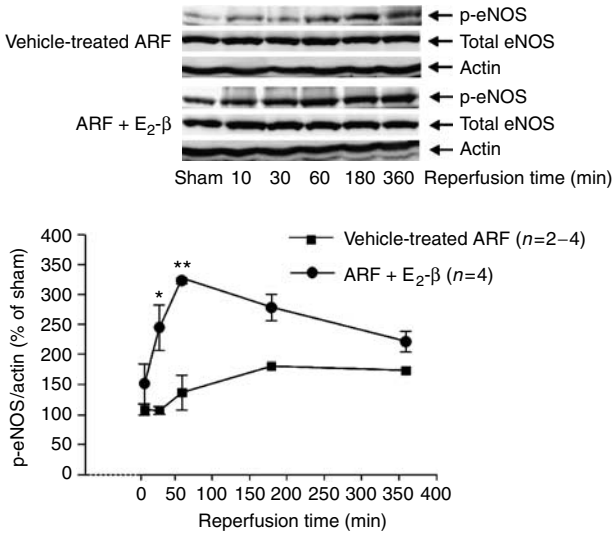

Figure 5 | Time course of total eNOS and phosphorylated-eNOS (p-eNOS, Ser^{1177}) expression in the kidney of vehicle-treated or E_2-β-treated ARF rats. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion. Mean density values are presented as fold difference compared with sham-operated rats. Each point and bar represents the mean ± s.e.m. *$P<0.05$, **$P<0.01$, compared with vehicle-treated ARF rats.

Table 3 | Effects of E_2-β on cNOS activity in post-ischemic kidney

Reperfusion time (min)	Vehicle-treated ARF (% of sham)	ARF+E_2-β
30	101.3 ± 7.7	134.7 ± 13.2
60	135.6 ± 10.5	182.9 ± 11.5*

ARF, acute renal failure; cNOS, constitutive NOS; E_2-β, 17β-estradiol.
Each value represents the mean ± s.e.m. of cNOS activity as fold difference compared with sham-operated rats. Inducible NOS activity was not detected in all groups. *$P<0.05$, compared with vehicle-treated ARF rats.

Effects of E_2-β on renal function after the I/R in wild-type mice and eNOS$^{-/-}$ mice

Renal function (BUN and Pcr) of wild-type mice subjected to 45-min ischemia and reperfusion showed a marked deterioration when measured 48 h after reperfusion. Administration of E_2-β to ARF mice 15 min before reperfusion markedly attenuated the I/R-induced renal dysfunction. eNOS$^{-/-}$ mice also showed the same degree of I/R-induced renal dysfunction, but this renal damage was not improved by treatment with E_2-β (Figure 11).

Effects of E_2-β on renal function after the I/R in bilateral ischemia model

In separate experiments, the effects of E_2-β on renal dysfunction induced by the I/R were examined using a bilateral ischemia model without prior uninephrectomy. As shown in Figure 12, renal function (BUN and Pcr) of rats subjected to 45-min ischemia and reperfusion showed a similar deterioration when measured 1 day after reperfusion in cases of one kidney model. The administration of

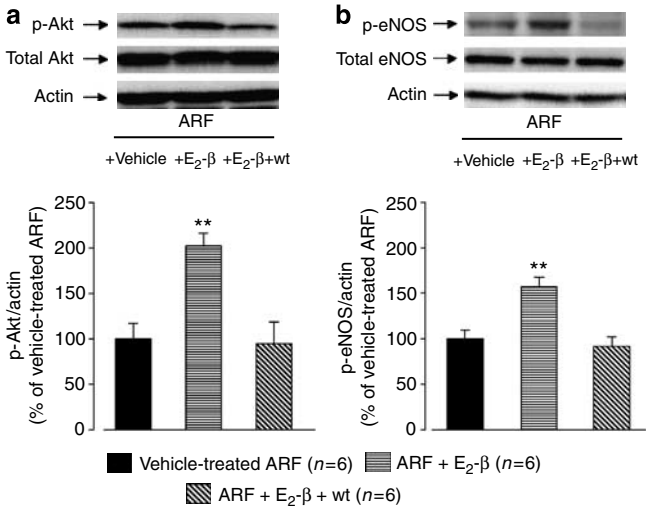

Figure 6 | Effects of E_2-β and wortmannin (wt) on phosphorylated-Akt (p-Akt, Ser^{473}) and phosphorylated-eNOS (p-eNOS, Ser^{1177}) expression in the kidney 30 min after reperfusion. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and wortmannin (10 μg kg^{-1}, i.v.) was given 10 min before ischemia. (**a**) Representative western blots for p-Akt, total Akt, and actin. (**b**) Representative western blots for p-eNOS, total eNOS, and actin. Mean density values are presented as fold difference compared with vehicle-treated ARF rats. Each column and bar represents the mean ± s.e.m. **$P<0.01$, compared with vehicle-treated ARF rats.

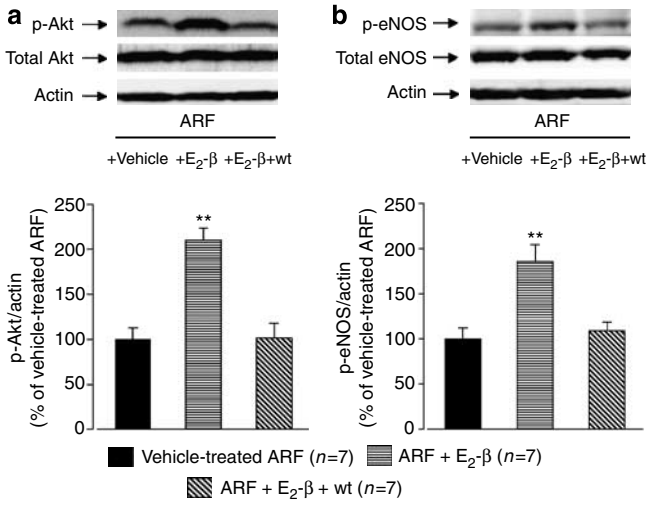

Figure 7 | Effects of E_2-β and wortmannin (wt) on phosphorylated-Akt (p-Akt, Ser^{473}) and phosphorylated-eNOS (p-eNOS, Ser^{1177}) expression in the kidney 60 min after reperfusion. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and wortmannin (10 μg kg^{-1}, i.v.) was given 10 min before ischemia. (**a**) Representative western blots for p-Akt, total Akt, and actin. (**b**) Representative western blots for p-eNOS, total eNOS, and actin. Mean density values are presented as fold difference compared with vehicle-treated ARF rats. Each column and bar represents the mean ± s.e.m. **$P<0.01$, compared with vehicle-treated ARF rats.

E_2-β 15 min before reperfusion at lower dose (10 μg kg^{-1}, i.v.) significantly attenuated the I/R-induced renal dysfunction, but higher doses (20, 100 μg kg^{-1}, i.v.) failed to improve it.

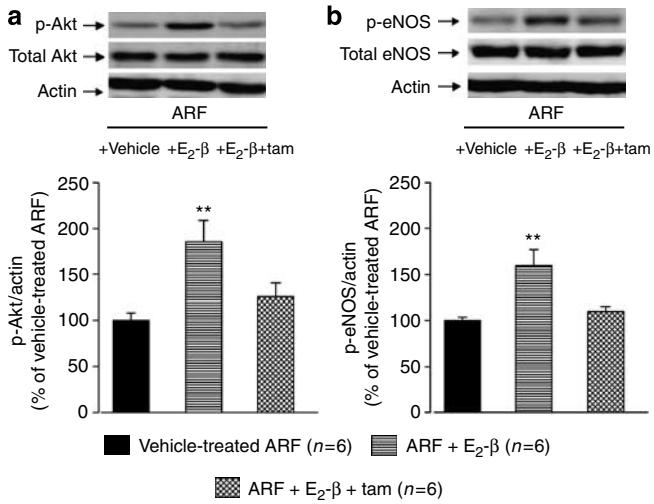

Figure 8 | Effects of E_2-β and tamoxifen (tam) on phosphorylated-Akt (p-Akt, Ser^{473}) and phosphorylated-eNOS (p-eNOS, Ser^{1177}) expression in the kidney 30 min after reperfusion. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and tamoxifen (5 mg kg^{-1}, i.v.) was given 60 min before ischemia. (**a**) Representative western blots for p-Akt, total Akt, and actin. (**b**) Representative western blots for p-eNOS, total eNOS, and actin. Mean density values are presented as fold difference compared with vehicle-treated ARF rats. Each column and bar represents the mean ± s.e.m. $^{**}P<0.01$, compared with vehicle-treated ARF rats.

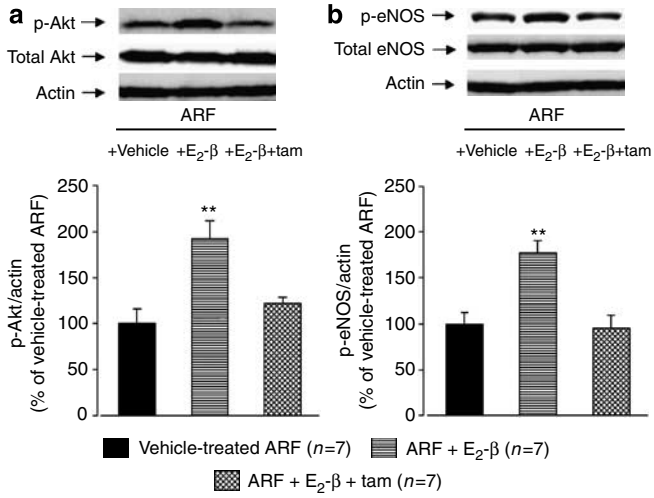

Figure 9 | Effects of E_2-β and tamoxifen (tam) on phosphorylated-Akt (p-Akt, Ser^{473}) and phosphorylated-eNOS (p-eNOS, Ser^{1177}) expression in the kidney 60 min after reperfusion. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion, and tamoxifen (5 mg kg^{-1}, i.v.) was given 60 min before ischemia. (**a**) Representative western blots for p-Akt, total Akt, and actin. (**b**) Representative western blots for p-eNOS, total eNOS, and actin. Mean density values are presented as fold difference compared with vehicle-treated ARF rats. Each column and bar represents the mean ± s.e.m. $^{**}P<0.01$, compared with vehicle-treated ARF rats.

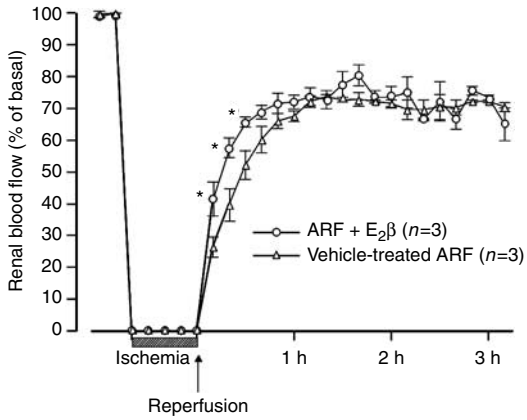

Figure 10 | Time course of renal blood flow in the post-ischemic kidneys with or without E_2-β treatment. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion. Each point and bar represents the mean ± s.e.m. $^{*}P<0.05$, compared with vehicle-treated ARF rats.

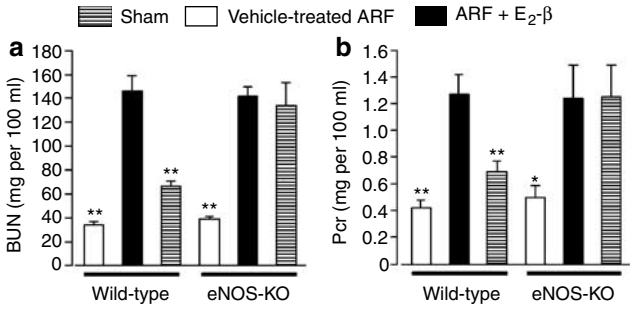

Figure 11 | Effects of E_2-β on (a) BUN and (b) Pcr 48 h after I/R in wild-type mice and eNOS$^{-/-}$ mice. E_2-β (100 μg kg^{-1}, i.v.) was given 15 min before reperfusion. Each column and bar represents the mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF mice.

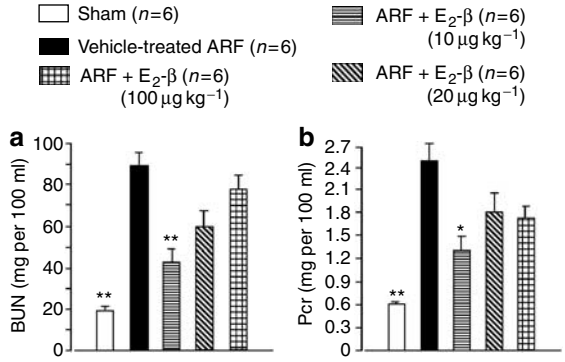

Figure 12 | Effects of E_2-β on (a) BUN and (b) Pcr 24 h after I/R in bilateral ischemia models. E_2-β (10–100 μg kg^{-1}, i.v.) was given 15 min before reperfusion. Each column and bar represents the mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF rats.

DISCUSSION

We have recently noted that the ameliorative effect of E_2-β against the post-ischemic renal injury is accompanied by increases in renal NO_x levels and that a non-selective NOS inhibitor can abolish the beneficial effects of E_2-β on the ischemic ARF.[14] Thus, the increasing effect of E_2-β on the renal NO production seems to be closely related to its

renoprotective effects. We and others have demonstrated that endogenous and/or exogenous NO protects the kidney from I/R-induced renal dysfunction and tissue injury.[10–13]

In this study, we investigated whether the PI3K/Akt pathway is positioned upstream to the enhancement of NO production in mechanisms underlying the renoprotective effects of E_2-β. Pretreatment with wortmannin, a PI3K inhibitor, or tamoxifen, an ER antagonist, abolished the E_2-β-induced renal protection observed 24 h after reperfusion. Furthermore, we found that E_2-β treatment markedly increased the levels of renal phosphorylated-Akt and phosphorylated-eNOS (Ser^{1177}) proteins in an early phase after reperfusion, and the augmentation of both proteins was also attenuated markedly by the combination with wortmannin or tamoxifen. Taken together, it seems likely that E_2-β activates the PI3K/Akt pathway, resulting in the increment of eNOS phosphorylation (Ser^{1177}) in the post-ischemic kidney, which plays a crucial role in the protective effects of E_2-β against I/R-induced renal injury. This view is supported by the findings that cNOS activities were significantly increased by E_2-β treatment at the same time points as seen in eNOS phosphorylation. Moreover, the treatment with E_2-β failed to improve the I/R-induced renal dysfunction in $eNOS^{-/-}$ mice, in contrast to the cases in wild-type mice, thereby suggesting that the eNOS component is essential to the renoprotective effect of E_2-β.

Mechanisms of increased production of NO by E_2-β have been reported in various studies.[19] One possible mechanism can be explained in part through the genomic action via ERs; when cultured endothelial cells[20–22] or cardiac myocytes[23] were exposed to E_2-β for 8–24 h, there were marked increases in NOS enzymatic activity and eNOS protein expression, accompanied by the elevation of eNOS mRNA levels, and these changes were abolished by ERs antagonists. In contrast, multiple pathways activated through the rapid, non-genomic action via membrane-associated ERs have been known to increase NO production. In endothelial cells, E_2-β caused a rapid (5–15 min) stimulation of eNOS activity, and this response was attenuated by an ERs antagonist but not by inhibition of gene transcription.[24] Simoncini *et al.*[16] have reported that ERα interacts with a subunit of PI3K and stimulates it, leading to the activation of Akt and eNOS. In our study, treatment with E_2-β rapidly enhanced the phosphorylation of Akt and eNOS (Ser^{1177}) and increased cNOS activities in the post-ischemic kidney, without changing total level of both proteins, suggesting the non-genomic action of E_2-β. However, we cannot rule out the possible involvement of the genomic mechanisms in the E_2-β-induced renal protection. A long-term effect of E_2-β on renal injury and eNOS mRNA levels in the post-ischemic kidney should be examined.

Our results are in agreement with the view that NO exerts a renoprotective effect against the I/R-induced renal injury.[12,13,25] We have previously noted that treatment with E_2-β in the ischemic ARF rats enhanced renal NO_x production in an early phase after reperfusion.[14] In this study, E_2-β treatment resulted in more rapid return of renal blood flow diminished by the ischemia. This restoration of blood flow seems likely to be due to the E_2-β-induced eNOS activation and NO production and may be at least partly contributive to the renoprotective effect of E_2-β. Moreover, we have noted that both exogenous and endogenous NO have protective effects against I/R-induced renal dysfunction and tissue injury, through the suppression of endothelin-1 overproduction,[26] which is known to be one of the major causal factors of this disease,[27,28] and that the improvement of ischemic ARF by E_2-β treatment was followed by the suppression of endothelin-1 overproduction in the post-ischemic kidney.[29] Most recently, we demonstrated that the pre-ischemic treatment with exogenous NO markedly suppressed the renal O_2^- production augmented by I/R, following the attenuation of neutrophil infiltration.[30] Neutrophil infiltration/migration appears to contribute to the post-ischemic ARF through various mechanisms.[31,32] Taken together, E_2-β given prior to the reperfusion may exert a renoprotective effect via NO-related multifunctional mechanisms.

In animal models, sex differences have been widely indicated in I/R injury in various organs, including the kidney. Müller *et al.*[6] demonstrated that kidneys of male mature rats are more susceptible to post-ischemic injury than those of females. Moreover, they found that castration and sexual immaturity did not affect the I/R injury produced in females but decreased the injury produced in males, thereby suggesting that testosterone plays a more important role in these sex differences than estrogen. However, E_2-β treatment of males but not testosterone treatment of females markedly improves the post-ischemic survival. Park *et al.*[5] also observed similar sex differences and E_2-β-induced beneficial action in male ARF rats. Furthermore, they demonstrated that testosterone administration increased susceptibility to renal I/R in females, ovariectomized females, and castrated males, in contrast to findings by Müller *et al.*[6] Thus, testosterone seems to play a crucial role in sex differences in I/R-induced renal injury, although there are some discrepancies. In contrast, we demonstrated the beneficial effect of E_2-β administration to male ARF rats and its signaling pathway. Further studies are needed to clarify whether estrogen is also an important determinant of the sex difference in renal I/R injury.

Clinical studies looking at outcomes in ARF patients have also shown that men have twice the mortality of women.[33,34] Despite improvements in intensive care and dialytic technology, there were no meaningful improvements in patient survival over the past three decades. The I/R-induced ARF is considered as an important determinant of allograft survival after renal transplantation. It is reported that the donor and/or recipient gender influences graft survival in the transplantation of some organs.[35,36] For example, a study dealing with the long-term survival outcomes of liver transplant recipients has shown that patient survival is significantly better in female recipients than in males.[36] Taken together, there is a possibility of E_2-β as a target for protective strategies to avoid ARF and to increase the rates of graft survival.

In our study, I/R was performed using uninephrectomized animals to investigate the renal dysfunction and tissue damage in one kidney; since collected urine is derived from one kidney, we can determine the renal excretory function of the kidney. Bilateral renal ischemia models have been generally used in a number of experiments, but uninephrectomized ischemia models are also well utilized. However, a previous study indicated that there are gender differences in remnant kidney growth, morphology, and function in rats two months after uninephrectomy.[37] Thus, one may doubt a possibility that the effects of E_2-β and related agents are influenced by uninephrectomy. Therefore, we evaluated whether E_2-β could attenuate the I/R-induced renal dysfunction in the bilateral ischemia model, and found that the lower dose (10 μg kg^{-1}, i.v.) significantly attenuated the I/R-induced renal dysfunction, but higher doses (20, 100 μg kg^{-1}, i.v.) failed to improve it. In our previous study,[29] E_2-β at 20–100 μg kg^{-1} produced a dose-dependent renoprotective effect in uninephrectomized ischemia models. The reason for the difference in the dose responses to E_2-β between bilateral and uninephrectomized models is unclear, but there is a report indicating that E_2-β treatment causes adverse effects on the renovasculature in a rodent model of renal injury.[38] In addition, studies examining the effects of E_2-β treatment on renal function have demonstrated both beneficial and adverse effects.[38–41] In contrast, there is convincing evidence indicating that testosterone is a more important factor than estrogen in the pathogenesis of bilateral I/R injury,[5] thereby suggesting the possibility that testosterone may mask the renoprotective effect of E_2-β in this model. Taken together, the renoprotective effects of E_2-β against post-ischemic renal injury may be dependent on differences in experimental conditions and models. Based on that bilateral ischemia is perhaps more relevant to the ischemic ARF injury in humans, further studies to evaluate the renoprotective effect of E_2-β and its mechanisms in the bilateral ischemia model are needed.

In conclusion, our findings using the uninephrectomized ischemia model demonstrate that E_2-β prevents I/R-induced renal injury by stimulating the PI3K/Akt pathway, resulting in the increment of phosphorylated-eNOS expression. Moreover, we indicated that the PI3K/Akt pathway is activated in an ER-dependent manner. The possibility that these renoprotective mechanisms of E_2-β are involved in sex differences in the ischemic ARF warrants further attention.

MATERIALS AND METHODS

Animals and experimental design

Rats. Male Sprague–Dawley rats (10 weeks of age; Japan SLC, Shizuoka, Japan) weighing 280–320 g were used. The animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed *ad libitum* access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg kg^{-1}, i.p.). After a 2-week recovery period, uninephrectomized rats were divided into five groups: (1) sham-operated control, (2) vehicle-treated ischemic ARF, (3) ischemic ARF treated with E_2-β (100 μg kg^{-1}, i.v.), (4) ischemic ARF treated with E_2-β (100 μg kg^{-1}, i.v.) and wortmannin (10 μg kg^{-1}, i.v.), and (5) ischemic ARF treated with wortmannin (10 μg kg^{-1}, i.v.). To induce ischemic ARF, the rats were anesthetized (sodium pentobarbital, 50 mg kg^{-1}, i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a non-traumatic clamp for 45 min. At the end of the ischemic period, the clamp was released for blood reperfusion.

In this study, the I/R was performed using uninephrectomized animals to investigate the renal dysfunction and tissue damage in one kidney; since collected urine is derived from one kidney, we can determine the renal excretory function of the kidney. Previous studies have been carried out in the same manner.[13,14,26,28–30] However, in some experiments, the effects of E_2-β (10–100 μg kg^{-1}, i.v.) on renal dysfunction induced by I/R were also examined using the bilateral ischemia model without prior uninephrectomy, in view of a possibility that the effects of E_2-β are influenced by uninephrectomy.

E_2-β or vehicle (a mixture of 2.5% ethanol, 30% polyethylene glycol 400, and 67.5% saline) in a volume of 1 ml kg^{-1} was injected 15 min before reperfusion. Wortmannin or vehicle (2.5% dimethyl sulfoxide and 97.5% saline) was injected 10 min before ischemia. In sham-operated control rats, the kidney was treated identically, except for the clamping. Animals exposed to 45 min ischemia were housed in metabolic cages at 24 h after reperfusion, and thereafter 5-h urine samples were taken and blood samples were drawn from the thoracic aorta at the end of the urine collection period. The plasma was separated by centrifugation. These samples were used for measurements of renal functional parameters. The kidneys were excised and examined using a light microscope.

In another set of experiments, we evaluated whether E_2-β-induced renoprotective effects were reversed by the combination with tamoxifen, an ERs antagonist. The experiments were performed in the same manner as described above. Briefly, uninephrectomized rats were divided into four groups: (1) sham-operated control, (2) vehicle-treated ischemic ARF, (3) ischemic ARF treated with E_2-β (100 μg kg^{-1}, i.v.), and (4) ischemic ARF treated with E_2-β (100 μg kg^{-1}, i.v.) and tamoxifen (5 mg kg^{-1}, i.v.). E_2-β was injected 15 min before reperfusion, and tamoxifen or vehicle (a mixture of 30% ethanol and 70% polyethylene glycol 400) in a volume of 1 ml kg^{-1} was injected 60 min before ischemia.

Moreover, in separate experiments, the left kidneys were obtained at 10, 30, 60, 180, and 360 min after I/R, frozen using liquid nitrogen, and used for western blot analysis and NOS activity measurements.

Mice. Male C57bl/6J wild-type and eNOS$^{-/-}$ mice (10 weeks of age; The Jackson Laboratory, Bar Harbor, ME, USA) weighing 20–25 g were used. Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg kg^{-1}, i.p.). After a 2-week recovery period, uninephrectomized mice were divided into three groups: (1) sham-operated control, (2) vehicle-treated ischemic ARF, and (3) ischemic ARF treated with E_2-β (100 μg kg^{-1}, i.v.). Experimental protocols and animal care methods in these experiments were the same as in cases using rats, described above. Animals exposed to 45-min ischemia were housed in metabolic cages at 24 h after reperfusion, and thereafter 24-h urine samples were taken and blood samples were drawn from the thoracic aorta at the end of the urine

collection period. These plasma and urine samples were used for measurements of renal functional parameters.

Renal functional parameters

BUN and creatinine levels in plasma (Pcr) or urine were determined using a commercial assay kit, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively. Uosm was measured by freezing point depression (Fiske Associates, Norwood, MA, USA). Urine and plasma sodium concentrations were determined using a flame photometer (Hitachi, 205D, Hitachinaka, Japan). Fractional excretion of sodium (FE_{Na}, %) was calculated from the formula $FE_{Na} = U_{Na}V/(P_{Na} \times C_{cr}) \times 100$, where $U_{Na}V$ is the urinary excretion of sodium and P_{Na} is the plasma sodium concentration.

Histological studies

Excised left kidneys were processed for light microscopic observation, according to the standard procedures. The kidneys were then fixed in phosphate-buffered 10% formalin, after which the kidneys were chopped into small pieces and embedded in paraffin wax, cut at 4 μm, and stained with hematoxylin and eosin. Histological changes were analyzed for proteinaceous casts, medullary congestion, and tubular necrosis, as suggested by Caramelo *et al.*[42] Tubular necrosis and proteinaceous casts were graded as follows: no damage (0), mild (1, unicellular, patchy isolated damage), moderate (2, damage less than 25%), severe (3, damage between 25 and 50%), and very severe (4, more than 50% damage). The degree of medullary congestion was defined by no congestion (0), mild (1, vascular congestion with identification of erythrocytes by $\times 400$ magnification), moderate (2, vascular congestion with identification of erythrocytes by $\times 200$ magnification), severe (3, vascular congestion with identification of erythrocytes by $\times 100$ magnification), and very severe (4, vascular congestion with identification of erythrocytes by $\times 40$ magnification). The scoring of the histological data was performed by independent observers in a double-blind manner.

Western blotting

The kidneys were washed in pre-cooled saline containing 5 mM EDTA, 2 mM β-glycerophosphate, 10 mM NaF, and 1 mM Na_3VO_4, frozen under liquid nitrogen, and homogenized with Multi-Beads Shocker (Yasuikikai, Osaka, Japan) in the buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 1 mM orthovanadate, 50 mM NaF, 20 mM β-glycerophosphate, 50 mM dithiothreitol, 1 mM PMSF (phenylmethanesulfonylfluoride), 50 kIU ml^{-1} aprotinine, 1 μg ml^{-1} pepstatin, and 2 μg ml^{-1} leupeptin). The homogenate was then centrifuged, stored at −80°C, and used for western blotting. Equal amount of protein was fractionated using 7.5 or 10% sodium dodecyl sulfate-polyacrylamide gels. After transfer to nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, USA), the blots were blocked for 60 min at room temperature with 5% nonfat dry milk in Tris-buffer saline containing 0.1% Tween 20 (for eNOS, Akt, and phosphorylated-Akt) or with Blocking One-P (Nacalai Tesque, Kyoto, Japan) containing 50 mM NaF (for p-eNOS). After blocking, the blots were incubated with primary antibodies (Akt, phosphorylated-Akt (Ser^{473}) and phosphorylated-eNOS (Ser^{1177} and Thr^{495}) from Cell Signaling Technology, Beverly, MA, USA; eNOS and actin from BD Biosciences, San Jose, CA, USA) at 4 °C overnight. Membranes were washed and then incubated with secondary antibodies (anti-rabbit horseradish peroxidase-linked IgG for phosphorylated-eNOS, Akt and phosphorylated-Akt from Zymed Laboratories, South San Francisco, CA, USA; anti-mouse horseradish peroxidase-linked IgG for eNOS and actin from Vector Laboratories, Burlingame, CA, USA) at room temperature for 60 min. Detection was carried out using an enhanced chemiluminescence western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and quantified by using the NIH IMAGE.

Measurement of NOS activity

Ca^{2+}-dependent and -independent NOS activities (constitutive and inducible NOS activities, respectively) were determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline using an NOS detection kit (NOS detection kit BOX-2; Sigma-Aldrich, St Louis, MO, USA). Briefly, frozen kidneys were homogenized in a buffer containing protease inhibitors. Homogenates were centrifuged and supernatant was utilized for the measurement of NOS activities. The samples were incubated in assay buffer containing 0.323 μM L-[^{3}H]arginine, 600 μM $CaCl_2$, and 1 mM NADPH, in the presence of an excess amount of calmodulin, at 37 °C for 30 min. After stopping the reaction, the samples incubated in resin, centrifuged, and radioactive activates of L-[^{3}H]citrulline were measured using a liquid scintillation counter (TRI-CARB; Packard, Tokyo, Japan). To determine Ca^{2+}-independent NOS activity, the assay was conducted in the presence of 10 mM EDTA without $CaCl_2$. Ca^{2+}-dependent NOS (cNOS) activity was calculated by subtracting Ca^{2+}-independent NOS activity from total NOS activity.

Statistical analysis

Values are expressed as the mean ± s.e.m. Relevant data were processed by InStat (Graph-PAD Software for Science, San Diego, CA, USA). For statistical analysis, we used the unpaired Student's *t*-test for two-group comparison and one-way analysis of variance followed by Dunnett's tests for multiple comparisons. Histological data were analyzed using the Kruskal–Wallis non-parametric test combined with Steel-type multiple comparison test. For all comparisons, differences were considered significant at $P < 0.05$.

ACKNOWLEDGMENTS

This work was supported by a 'High-Tech Research Center' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2002–2006, and a Grant-in-Aid for scientific research from MEXT.

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